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Award Number DAMD17-96-1-6060

TITLE: Antagonistic Action of Hyaluronan Oligomers in Breast Cancer

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REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (1 Sep 98 - 31 Aug 99)

4. TITLE AND SUBTITLE
Antagonistic Action of Hyaluronan Oligomers in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6060

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The objective of this project is to determine whether hyaluronan interactions are involved in growth and invasion of mammary carcinoma cells inascites. Syngeneic mice were injected intraperitoneally with stable transfectants of TA3/St murine mammary carcinoma cells that overexpress soluble CD44, a hyaluronan-binding protein that would be expected to compete with endogenous hyaluronan-protein interactions. The behavior of these transfectants was compared with that of transfectants expressing mutated soluble CD44 that does not bind hyaluronan. Overexpression of soluble CD44, but not mutated solble CD44, inhibited tumor cell growth in ascites as well as invasion of the peritoneal wall. When grown in vitro in soft agar, the soluble CD44 transfectants exhibited a dramatic reduction in colony formation compsrted to wild type, vector-transfected and mutant soluble CD44-transfected TA3/St cells. Thus, perturbation of hyaluronan interactions by soluble CD44 has a direct effect on the growth characteristics of these tumor cells, leading to inhibition of anchorage independent growth in vitro and ascites growth in vivo.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Murine Mammary Carcinoma Hyaluronan, CD44, Invasion	8
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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PI - Signature 12/30/99 Date

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(5) INTRODUCTION

The objective of this project is to determine whether hyaluronan interactions are involved in growth and invasion of mammary carcinoma cells in ascites. Hyaluronan accumulates in ascites during intraperitoneal proliferation of TA3/St murine mammary carcinoma cells and at sites of their invasion of the peritoneal wall. To determine whether hyaluronan is functionally involved in these events, mice were injected intraperitoneally with stable transfectants of TA3/St cells that overexpress soluble CD44, a hyaluronan-binding protein that would be expected to compete with endogenous hyaluronan-protein interactions. The behavior of these transfectants was compared with that of transfectants expressing mutated soluble CD44 that does not bind hyaluronan. We have reported previously that the soluble CD44 transfectants temporarily grew at a reduced rate within the peritoneal cavity, then went into G1 arrest and were subsequently cleared from the peritoneum. However, the transfectants overexpressing mutant soluble CD44 that does not bind hyaluronan exhibited similar ascites accumulation, growth rates and cell cycle profiles in vivo to wild type and vector-transfected TA3/St cells, all of which continued to grow until the tumors became fatal. The soluble CD44-transfected TA3/St cells also failed to attach to and form tumors in the peritoneal wall. These experiments suggest that perturbation of hyaluronan interactions by soluble CD44 alters the growth characteristics of the tumor cells, leading to inhibition of ascites growth and invasion in vivo. However, it was not clear whether overexpression of soluble CD44 has a direct effect on tumor cell behavior or whether its effect was an indirect consequence of another event in vivo. In the experiments reported here, we have begun to investigate whether inhibition of tumor growth is due to direct or indirect effects.

(6) BODY

Soluble CD44-Transfected TA3/St Mammary Carcinoma Cells Have Lost the Capacity for Anchorage Independent Growth In Vitro. Since it was not clear from the in vivo results obtained previously whether overexpression of soluble CD44 has a direct effect on tumor cell growth or whether its effect was an indirect consequence of another event in vivo, we sought additional evidence to discriminate between these two possible explanations.

First, proliferation of the soluble CD44 transfectants and control cells was compared in monolayer culture in tissue culture wells. Each cell line grew at approximately the same rate over a five day period and all cell lines exhibited similar cell cycle profiles (Table 1). This result was in contrast to the differences observed previously between the growth of soluble CD44 transfectants in ascites vs that of controls in ascites, where the former were found to go into G1 arrest (Table 1).

We then examined anchorage independent growth of the various cell lines in soft agar. Soft agar assays were performed in 60 mm dishes containing 2 ml of 1.2% agarose diluted with 2x DME medium containing 20% fetal bovine serum (FBS) to yield a final agarose concentration of 0.6%. Cells were harvested from monolayer culture in log growth phase by trypsinization, washing and resuspension in DME medium containing 10% FBS for

counting. The cells were then suspended in 0.33% agarose in DME medium containing 10% FBS, with or without 0.5 mg/ml Geneticin, and plated at 5000 cells/ well on top of the 0.6% agarose base. After each agarose layer was allowed to solidify (10 min at 25°), three additional 1 ml volumes of 0.33% agarose were layered on top of the cells. Each cell line was plated in triplicate and grown at 37° for 28 days. Total numbers of colonies per well containing >30 cells or >200 cells per colony were counted separately using a microscope grid. The two classes of colony size were assessed by counting cells in numerous colonies under the microscope and correlating these numbers with colony size. The two classes could be distinguished readily since the great majority of colonies were found to contain between 30 and 100 cells; the large colonies (>200 cells) were very easily distinguished from the majority of colonies (30-100 cells) and there were virtually no colonies with <30 cells.

Dramatic differences in size and number of colonies formed between the soluble CD44 transfectants and control cells were observed (Table 2). The wild type, vector-transfected cells and mutant soluble CD44-transfected cells formed many times more colonies than the soluble CD44 transfectants, and the colonies formed by the control cells were much larger than those few colonies formed by the soluble CD44 transfectants (Table 2).

Thus we have shown that the soluble CD44 transfectants, but not the mutant soluble CD44 transfectant, have lost their ability to exhibit anchorage independent growth in soft agar, one of the most reliable indicators for the transformed state of cells. We conclude that endogenous hyaluronan produced by the tumor cells themselves directly serves an important function in anchorage independent growth, and that hyaluronan interactions at the cell surface are, at least under some circumstances, crucial to mammary cancer cell growth characteristics in vitro and in vivo.

Table 1 Soluble CD44 transfectants of mammary carcinoma cells exhibit G1 arrest in vivo but not in vitro

Cell type	% Cells in G0/G1	
	In vitro	In vivo
Controls:		
Wild type TA3/St	44.1±0.5	30.0±2.1
Vector transfectant	28.3±1.5	38.2±5.4
Soluble CD44 v6-v10 R43A[a]	33.3±0.9	39.1±4.8
Soluble CD44 Transfectants:		
Soluble CD44 v6-v10a	44.7±1.6	86.2±1.0
Soluble CD44 v6-v10b	37.0±2.1	76.4±4.1
Soluble CD44 v8-v10	53.0±1.2	74.3±2.8

[a]The R43A clone expresses mutated soluble CD44 that does not bind hyaluronan

Table 2 Soluble CD44 transfectants of mammary carcinoma cells fail to form colonies in soft agar

Cell type	Number of colonies[a]	
	>30 cells	>200 cells
Controls:		
Wild type TA3/St	205±28	30±2
Vector transfectant	83±21	25±9
Soluble CD44 v6-v10 R43A[b]	409±11	39±5
Soluble CD44 Transfectants:		
Soluble CD44 v6-v10a	1±0	0±0
Soluble CD44 v6-v10b	16±4	5±1
Soluble CD44 v8-v10	8±1	0±0

[a]Numbers represent means (±SD) of the total numbers of colonies with >30 or >200 cells per colony in triplicate wells.
[b]The R43A clone expresses mutated soluble CD44 that does not bind hyaluronan

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Completion of Ph.D. requirements by Rebecca Moore Peterson. Rebecca Peterson has now completed all requirements for the Ph.D. in Cell, Molecular and Developmental Biology at Tufts University and has been awarded the Ph.D. She is currently employed as an Instructor in Biology at Penn State University.

Transfer of award to Jeanine Ward. This award has now been transferred to Ms Jeanine Ward. Ms Ward will continue the above work, utilizing additional approaches to determine the effect of hyaluronan on tumor cell growth and invasion.

(7) APPENDICES

Key research accomplishments:

a. Demonstration that hyaluronan interactions are crucial to growth of murine mammary carcinoma cells in ascites

b. Demonstration that hyaluronan interactions are crucial to invasion of murine mammary carcinoma cells from ascites into the peritoneal wall

c. Demonstration that hyaluronan interactions are crucial to anchorage independent growth of murine mammary carcinoma cells in vitro

Outcomes:

a. Manuscript in preparation reporting above results

b. Ph.D. in Cell, Molecular and Developmental Biology at Tufts University awarded to Rebecca Peterson

c. Award of National Cancer Institute grant (RO1 CA82867) to pursue work based in part on results described above